MOTILITY AND AGING OF ARBACIA SPERM [1]

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It is well known that diluted sea urchin sperm have a relatively short effective life span. In a few hours or days they lose the ability to activate eggs, become immotile and their respiration ceases (Gemmill, 1900; Cohn, 1918; Gray, 1928, 1931; Rothschild, 1951; Tyler, 1953; Rothschild and Tyler, 1954; Bishop, 1962; Mann, 1964).

The loss of vitality of sperm is reported to be associated with the exhaustion of energy reserves (Gemmill, 1900; Cohn, 1918; Tyler, 1953). Gemmill recognized that sperm were more active, and also pointed out (p. 171) that "on comparing the movement of spermatozoa in different mixtures [dilutions], one finds that the difference in activity is not sufficiently marked to account for the very early loss of vitality of spermatozoa in the weaker mixtures simply in terms of exhaustion of energy." The relationship between the motility of sea urchin sperm and the rate at which they lose the ability to activate eggs remains somewhat uncertain, primarily because it is difficult to evaluate quantitatively the motility of spermatozoa.

The problems of finding the motility status of semen samples are manifold. Microscopic examination to determine sperm activity involves many variables that are difficult to control (Bishop, 1962; Rikmenspoel, 1962; Rothschild, 1953; van Duijn, 1963, 1964). The impedance change frequency method for rating motility (Rothschild 1948a) allows better control of these variables but is unsuitable with diluted semen. A simple method of rating sperm motility which avoids some of these problems was devised for this study.

The first part of this report deals with an analysis of the method for rating motility. The second part is an investigation of senescence of sperm, utilizing the method for rating motility. The following results show that under the conditions of these experiments the concentration, motility and fertility of sperm suspensions diminished most rapidly soon after dilution and more slowly later on. The rapid initial decline in fertility and concentration was prevented by experimentally immobilizing the spermatozoa, but immotile semen ultimately lost fertility at about the same time as motile semen.

Material and Methods

Arbacia punctulata was furnished by the Supply Department of the Marine Biological Laboratory at Woods Hole, Mass. Gametes were obtained by electrical stimulation of the intact animals (*cf.* Costello *et al.*, 1957). Sperm were collected

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in 100- or 250-ml. beakers of sea water by immersing the aboral surface of the urchin, along with one electrode of the stimulator, and applying the other electrode to the oral surface. Sperm accumulated in piles on the bottom of the beaker in a relatively undiluted state. The time of the first stirring of the sperm into suspension was considered the beginning of the experiment. Eggs were shed in a similar fashion into 100 ml. of sea water, but only a few thousand at a time so that the same female could be used repeatedly to produce fresh eggs.

Sperm concentration was determined by counting in a Neubauer hemocytometer and/or by measuring light absorption with a Klett-Summerson colorimeter (green filter, *cf.* Iverson, 1964).

Fertilizing capacity was determined by diluting sperm in two-fold steps, then adding about 500 eggs to each dilution (total volume 5 ml.). Fertilizing capacity was rated numerically by taking the reciprocal of the sperm concentration at which less than 100% cleavage was attained and above which all eggs were fertilized.

Motility was rated by comparing the sedimentation of living and formalin-killed portions of sperm suspensions. Sedimentation was enhanced by motility. In order to rate motility experimentally, two 15-ml. portions of diluted sperm suspensions (usually about 20 million cells/ml.) were withdrawn and one portion killed with 0.02% formalin. Sperm concentration of the two portions was determined by optical density (O.D.) measurements. Both samples were then centrifuged at 200 *g*, 20° C. for 20 minutes in conical tubes in swinging buckets. After centrifugation 5-ml. portions of the supernatant were withdrawn from one centimeter above the bottom of the tubes and their O.D. determined. The difference between the O.D. before and after centrifugation was proportionate to the number of sperm sedimented. This difference was always greater for suspensions of motile sperm. The difference between the decrease in O.D. of live and formalin-killed suspensions was assumed to result from the "downward" migration of sperm in the motile sample, and was considered to be the motility score of the sample (*cf.* van Duijn, 1963, for discussion of sperm migration rate). This score was determined by the equation

$$M = dL - dK,$$

where M is the motility score, dL is the change in O.D. ($\times$ 100) of the living sample, presumably resulting from sedimentation plus "downwards" swimming of sperm, and dK is the change in O.D. ($\times$ 100) of the formalin-killed sample representing sedimentation unaltered by motility. Under these conditions the value of M for 50 freshly diluted sperm varied from 3 to 41 with a mean value of 14, with 15 recurring most frequently.

Experiments and Results

Motility by centrifugation

Motility can be rated quantitatively by finding measurable differences between motile and immobile samples of sperm that are otherwise equivalent. It was found that such differences resulted when live and dead sperm suspensions were centrifuged. Living, motile sperm sedimented faster than formalin-killed ones, and the difference could be measured as described above. A possible explanation of the

difference in sedimentation rates is that sperm oriented head "downwards" in the centrifugal field (*cf.* Rothschild, 1962) because their tails are more buoyant than their heads (Kihlström, 1958; Beatty, 1964), and, therefore, motile sperm swam "downwards" faster than dead ones sank.

The hypothesis that sperm were oriented by centrifugal force was tested in the centrifuge microscope. Formalin-killed sperm were seen to be oriented head "downwards." Living sperm moved very rapidly "downwards," but it was impossible to see any orientation because of the rapid movement of the sperm superimposed on the flashing field of the microscope, and so it remained uncertain whether or not living sperm were oriented in the centrifugal field.

The following experiments tend to support the assumption that living sperm sedimented faster than dead ones because they swam "downwards." Sperm from the pellet that formed when living sperm were centrifuged were highly active

TABLE I

*Motility and fertilizing capacity of various fractions of centrifuged sperm**

Experiment number	Motility**		Fertilizing capacity***		
	Pellet	Control	Pellet	Supernatant	Control
1	9	3	0.22	0.03	0.21
2	9	4	5.0	0.13	2.5
3	15	4	2.5	1.4	—

* Sperm were centrifuged 20 minutes at 200 *g*. The upper 5 ml. of the supernatant and the sperm in the pellet were then withdrawn by pipette. Pellet sperm were resuspended in sea water to the original concentration. These fractions were then tested for motility or fertilizing capacity. The original uncentrifuged suspension served as a control for aging.

** Motility score (M) is in Klett units.

*** Fertilizing capacity is the reciprocal of sperm concentration wherein just less than 100% fertilization was attained ($\times 10^{-6}$).

when viewed under a microscope. Sperm remaining in the supernatant seemed less active. When sperm from the pellet were resuspended in sea water and tested for motility by centrifugation, they had higher motility scores than control sperm of the same age, and were more effective at fertilizing eggs than either sperm from the supernatant or uncentrifuged control sperm (Table I). This could mean that sperm were improved by being packed into a pellet, or, as seems more likely, that the most active and effective ones were concentrated by centrifugation.

The experiments on aging reported below also tend to confirm that the difference in sedimentation rates resulted from motility. The proportion of live sperm sedimented (dL) was greatest at first when samples were visibly most active and gradually decreased until it equalled the proportion of dead sperm sedimented (dK) as the samples aged and became immotile (Fig. 1). Similarly, sperm immobilized at low pH or by narcosis with carbon dioxide (*cf.* Mohri and Yasumasu, 1963) sedimented at the same rate as formalin-killed sperm: that is to say, hardly at all (Fig. 2). It therefore seems likely that the formalin-killed sperm sedimented more

slowly than live ones because their motility was inhibited rather than because of some extraneous effect of the formalin.

Motility rating by centrifugation could be influenced by the sperm concentration (Rothschild, 1956a; Tampion and Gibbon, 1963). Table II compares the values obtained when the motility of the same sperm sample was determined at different concentrations. Sometimes, but not always, the more dilute sperm gave lower motility values. This may have resulted from more rapid aging by more dilute sperm (Rothschild, 1948b). Dilute sperm tended to lose motility very rapidly at first (Fig. 1), but as Gray (1928) observed, sea urchin semen samples vary. He reported that the respiration of some samples declined rapidly after dilution, while others showed some lag before beginning to decline. The concentration effect on

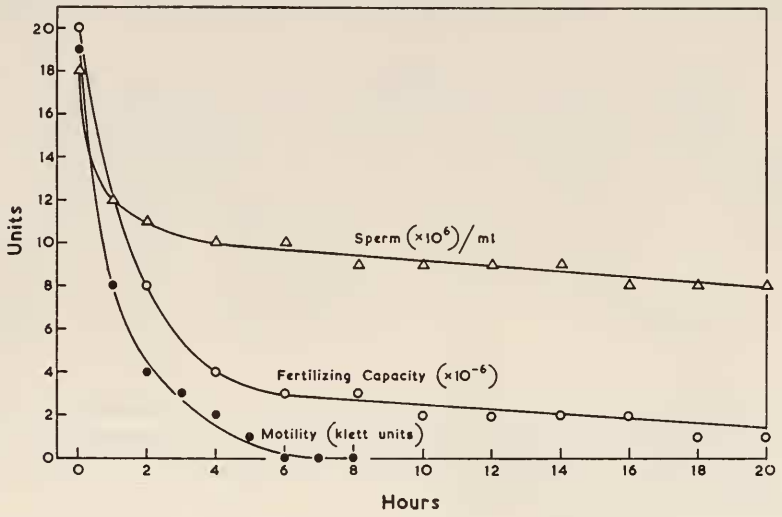

FIGURE 1. Change in sperm concentration, fertilizing capacity and motility with time. Initially *Arbacia* semen was diluted to 18×10^6 cells/ml. in 500 ml. of sea water. Periodically the suspension was stirred and portions tested for motility and fertilizing capacity. Motility is in Klett units. Fertilizing capacity is expressed as the reciprocal of the concentration wherein just less than 100% fertilization was attained (units $\times 10^{-6}$). Sperm concentration is in millions of sperm/ml. (units $\times 10^6$).

motility rating that was sometimes observed may also have reflected physical interaction between sperm (Taylor, 1952; Rikmenspoel, 1962; Tampion and Gibbon, 1963; van Duijn, 1963). The motility score used in the experiments reported below is probably valid only if sperm concentration and time after dilution are taken into account.

Sperm senescence

This somewhat quantitative method for rating motility was used to investigate the relationship between motility and the loss of fertilizing capacity by aging sea urchin spermatozoa.

In the initial experiments semen was diluted about 2000-fold in filtered sea water

and allowed to stand in 100- or 250-ml. beakers at room temperatures. Periodically the suspensions were thoroughly stirred and portions tested by centrifugation for motility, and in serial dilution for fertilizing capacity. Sperm concentration was determined by absorptiometry at each interval and confirmed occasionally by direct counts.

Under the conditions of these experiments (25 in all) sperm concentration as well as motility and fertilizing capacity declined with time (Fig. 1). In different

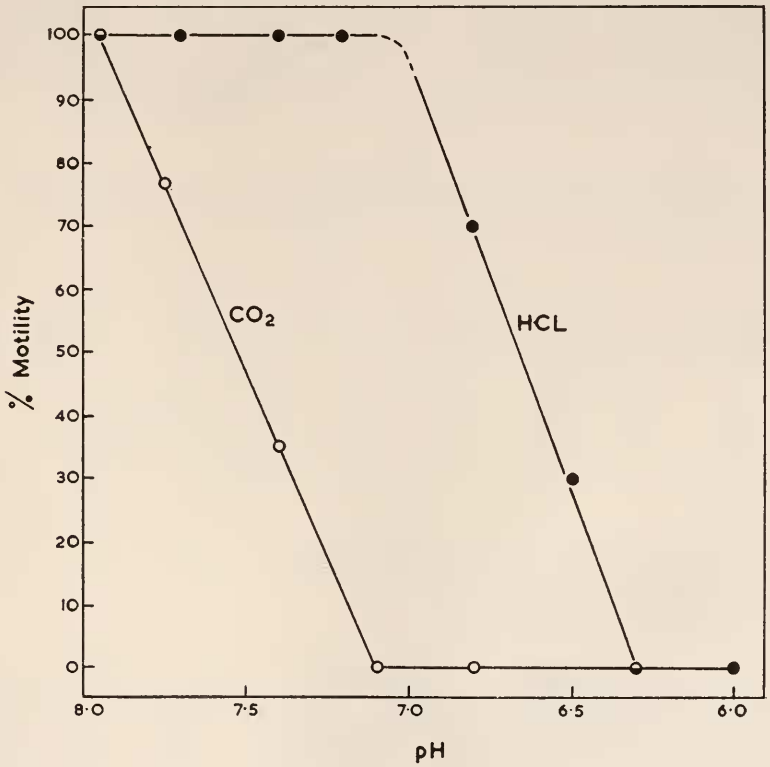

FIGURE 2. Effects of acid and carbon dioxide on *Arbacia* sperm motility. Sperm were shed into 250 ml. of sea water to 18×10^6 sperm/ml., then divided into three equal portions. One portion served as control, the second was acidified by bubbling with 6.8% CO_2 in air, and the third was acidified with HCl. The motility of the three portions was determined simultaneously by centrifugation. The process was repeated at different pH values with fresh sperm shed from the same male. The data are presented as percentage of the control sperm motility.

samples the average rate of decrease in concentration varied from 0.25×10^6 sperm/ml. lost each hour to 5×10^6 sperm/ml. lost each hour. There was no evidence that the loss of sperm resulted from sedimentation or the adherence of sperm to the walls of the container. The decrease in sperm concentration had to be considered in motility and fertilization capacity determinations, so concentration was determined and new portions killed for motility determination at each time interval.

Motility and fertilizing capacity of sperm declined most rapidly soon after dilution and more slowly later on (Fig. 1). Motility became imperceptible, as rated by the centrifuge method, after about six hours. Such "immobile" sperm were seen under the microscope to be twitching slightly but not progressing. Fertilizing capacity, on the other hand, persisted for 30 to 40 hours after dilution (as determined directly in three experiments and estimated from semilog plots of the data from the other experiments). Actively moving sperm were observed trapped in the jelly coat of eggs inseminated with dilutions of aged, apparently immotile sperm. This suggested that sperm were stimulated to renewed activity under the conditions of fertilization. The possibilities were considered that such rejuvenation could have resulted from (1) further dilution (Rothschild, 1956a) or (2) stimulation by substances exuding from eggs (Hathaway, 1963).

TABLE II

*The effect of sperm concentration on motility determination by centrifugation**

Dilution		Sperm sample								
		1	2	3	4	5	6	7	8	9
1	C**	22	33.5	61	36	47	19	31	17.5	19
	M***	15	17	7	6	25	19	7	15	15
2	C	11	20.5	29	18	18	14	16.5	16	17
	M	15	6	6	6	6	15	7	9	15
3	C	—	9.5	13	9	8	11	7.5	14	16
	M	—	6	3	6	4	8	7	10	17

* Semen was serially diluted and tested for motility.
** Concentration is in millions of sperm per ml.
*** M = Motility score in Klett units.

Sea urchin sperm are known to be stimulated to a burst of activity by dilution in sea water (*cf.* Mann, 1964, p. 343). The rejuvenation of sperm suggested above could have resulted from such a "dilution effect." Sperm activity in the aged samples could have been suppressed by exhaustion of oxygen or accumulation of respiratory CO_2 with a concomitant fall in pH (Rothschild, 1956a; Mohri and Yasumasu, 1963), and then restored by further dilution. In order to test this hypothesis, air was bubbled through sperm suspensions as they aged (two experiments). Aerated sperm lost motility at a uniform rate while the control sperm lost motility more rapidly at first and more slowly later on. Some motility persisted in the control samples for several hours after the aerated ones lost perceptible motility. Concentration and fertilizing capacity declined more rapidly in aerated than in control suspensions. The pH was constant (8.0) in the aerated sample but fell to pH 7.6 in the controls during the first hour, and then remained constant. Rothschild (1956b) reported that a pH increase from 7.84 to 8.00 resulted in a 400% increase of the respiratory rate of *Echinus esculentus* sperm. Mohri and Horiuchi (1961), on the other hand, reported that Japanese sea urchin sperm (*Hemicentrotus pulcherrimus, Pseudocentrotus depressus* and *Anthocidaris crassispina*) were little

affected by varying the pH from 7.0 to 8.5. In order to evaluate the significance of the observed drop in pH on *Arbacia* sperm suspensions the effects of acid and carbon dioxide on motility were compared with the centrifuge method for rating motility. In three experiments the hydrogen ion concentration of freshly shed sperm suspensions was adjusted to a given pH in the range of 8.0 to 6.0, either with HCl or by bubbling 6.8% CO_2 in air through the suspension. The motility of acidified sperm was then compared with control sperm and results expressed as the percentage of control motility remaining (Fig. 2). The results clearly indicated that CO_2 inhibited motility at hydrogen ion concentrations which had little or no effect on *Arbacia* sperm motility. The fall in pH from 8.0 to 7.6 observed in the preceding experiments on aging could have appreciably suppressed motility if it resulted from accumulating CO_2.

It was of interest to see if the fertile life of diluted sea urchin sperm could be prolonged by depressing their motility (*cf.* VanDemark, Koyama and Lode, 1965). It has long been known that sperm immobilized with acid or CO_2 can fertilize eggs after dilution in sea water (Cohn, 1918). In four experiments sperm were diluted and allowed to age in sea water adjusted to pH 6.5 or 7.0 with HCl or CO_2. The pH was adjusted occasionally so that it did not fluctuate more than 0.1 unit from the desired value. Periodically samples were tested for sperm concentration and fertilizing capacity. In these experiments the fertilizing capacity of acidified sperm remained at the high initial value for about 24 hours and then declined rapidly so that all sperm became infertile after about 32 hours. Control sperm (in sea water, pH 8.0–7.8) lost fertilizing capacity rapidly in the first few hours and then more slowly until all fertilizing capacity was lost at about 32 hours (*cf.* Fig. 1). In one experiment sperm were completely immotile in sea water adjusted to pH 6.5 with either HCl or CO_2 (*cf.* Fig. 2). The fertilizing capacity of both acidified samples was initially about 25% less than the control value, but persisted undiminished for 22 hours and then declined rapidly in both samples. At pH 7.0 (three experiments) sperm in sea water with HCl were slightly motile while those in sea water with CO_2 were immotile. The fertilizing capacity of both samples remained the same as the initial control value until the rapid decline began after about 24 hours. The fertilizing capacity of sperm in HCl-adjusted sea water began to decline about two hours before the ones in CO_2-adjusted sea water in all three experiments. In these experiments the sperm concentration diminished less rapidly in the acidified suspensions than in the control suspensions. In one experiment the control sperm were all gone after 14 hours, while the concentration of sperm held at pH 7.0 did not diminish in 32 hours. In a fifth experiment 50 units of penicillin per ml. were used to control bacteria (Mohri, 1957). In this six-hour experiment, cell loss was reduced by penicillin to the level observed in the immobilized suspensions, while the concentration of sea water control sperm diminished 0.26 million cells per ml. hour. The penicillin had a marked detrimental effect on motility and fertilizing capacity, however, so the experiment was discontinued.

A second hypothesis accounting for the rejuvenation of aged sperm is that substances exuding from eggs could reactivate "immobile" sperm (Hathaway, 1963), just as the surface of the chorion near the micropyle of fish eggs activated motionless sperm (Yanagimachi, 1957). It proved unfeasible to test this hypothesis, however, because the water in which eggs had stood caused irreversible agglutination

of formalin-killed sperm (*cf.* Tyler and Bishop, 1963) and thus rendered motility rating by the centrifuge method unreliable.

Conclusions

Senescence of *Arbacia* sperm in the experiments reported above occurred in several phases. Soon after dilution, sperm motility, fertilizing capacity and concentration declined rapidly. After about six hours motility became imperceptible by the rating method used here. Thereafter the concentration and fertility of the remaining sperm declined much more slowly (Fig. 1). Ultimately after 30 to 40 hours, all fertility was lost.

Fertility and sperm concentration both declined most rapidly soon after dilution when motility was greatest (Fig. 1). This initial rapid decline was prevented by suppressing motility with hydrogen ions and/or carbon dioxide. It therefore seems likely that the loss of sperm from suspension and the initial diminution of fertility were both associated with exhaustion of energy supplies. Afzelius and Mohri (1966) demonstrated that reduction in mitochondrial cristae apparently resulted from the catabolism of phospholipids and suggested that sea urchin sperm might burn up structural elements for energy. It is conceivable that the observed decrease in sperm concentration resulted from such autolysis.

Bacterial contamination could have been involved in the destruction of sperm and the loss of fertility by the remaining ones. The decline in sperm fertility and concentration, however, was most rapid soon after dilution when bacterial contamination should have been minimal, and the rate of decline diminished later when bacterial effects should have been more pronounced. It seems more likely that bacteria had a role in the final phase of sperm aging, when all fertility disappeared from the samples, rather than in the striking initial changes. This final loss of fertility was probably due to factors other than exhaustion of energy reserves, since it occurred at about the same time whether motility was suppressed or not (*cf.* Mann, 1964, p. 349).

Gray (1931) emphasized that sperm suspensions are heterogeneous populations of cells. He recognized this heterogeneity in his data as the varying rate of decline of respiration as sperm aged, and attributed it to physiological variability in the composition of individual cells. The data presented above can also be interpreted in this way.

Figure 1 suggests that the semen samples consisted of a population of short-lived sperm that disappeared during the first phase of aging and a second population that persisted through the second phase. Perhaps the short-lived sperm exhausted their energy supplies rapidly while the second population maintained a reserve of energy. Slow-speed centrifugation is apparently a useful method for separating sperm according to motility. It therefore presents a tool to investigate further physiological variability within semen samples and the significance of this variability to studies of gamete physiology and development.

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Hathaway for a pressure siphon used for withdrawing samples from centrifuge tubes while leaving the sediments undisturbed.

Summary

1. The aging of semen was investigated in terms of sperm motility. A method for rating motility was devised. It was based on the observation that motile sperm sedimented faster than formalin-killed controls when subjected to low-speed centrifugation.

2. The aging of semen was found to involve the loss of motility, the loss of fertilizing capacity and disappearance of sperm from suspension. All three of these factors declined most rapidly at first and more slowly later on. The rapid loss of fertilizing capacity and the disappearance of sperm could be delayed by suppressing motility with hydrogen ions or carbon dioxide.

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